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Therapeutic proteins: a comparison of chemical and biological properties of uricase conjugated to linear or branched poly(ethylene glycol) and poly(*N*-acryloylmorpholine)

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Abstract

Uricase from *Bacillus fastidiosus* (UC) was covalently linked to linear PEG (PEG-1) (M_w 5 kDa), branched PEG (PEG-2) (M_w 10 kDa) and to poly(*N*-acryloylmorpholine) (PAcM) (M_w 6 kDa). The conjugation of UC with linear PEG and PAcM was accompanied by complete loss of enzymatic activity but, if uric acid as site protecting agent was included in the reaction mixture, the conjugate protein retained enzymatic activity. On the other hand, the modification with PEG-2 gave a conjugate that also maintained enzymatic activity in the absence of any active site protection. This behaviour must be related to hindrance of the branched polymer in reaching the enzyme active site. The UC conjugates exhibited increased resistance to proteolytic digestion while minor variations in the inhibitory constant, optimal pH, heat stability, affinity for substrate, were observed. Pharmacokinetic investigations in mice demonstrated increased residence time in blood for all the conjugates as compared with native uricase. Uricase conjugated with linear PEG was longer lasting in blood UC derivative, followed by branched PEG and the PAcM conjugates. Unconjugated uricase was rapidly removed from circulation. All these data are in favour of the use of the less known amphiphilic polymer PAcM as an alternative to PEGs in modification of enzymes devised for therapeutic applications.

Keywords: PEGylation; PEG; Branched PEG; Poly(*N*-acryloylmorpholine); Uricase; Therapeutic proteins

1. Introduction

Uricase (urate oxidase EC 1.7.3.3, UC) catalyses the oxidation of uric acid, a final product of purine catabolism, to allantoin which is more soluble and more easily excreted than the starting compound. The enzyme is a tetramer composed of two types of different subunits with a final molecular weight in the range 145–150 kDa [1–3]. The subunit size, as calculated from the cDNA sequence, is 35 kDa [4]. The enzyme is widely present in most vertebrates whilst absent in man.

When uric acid increases in blood over normal values it can cause renal failure and may contribute to a group of diseases usually known as gout. Severe hyperuricemia is also a serious complication of hematologic malignancies and of antineoplastic chemotherapy [5]. To prevent these disorders, drugs have been employed that induce forced diuresis, urinary alkalinization as well as decreased synthesis of urate by xanthine oxidase administration. However, these treatments are sometimes ineffective and serious side effects may also occur. As an alternative treatment the administration of uricase was suggested, this rapidly lowers plasma urate levels [5–8]. However, repeated treatment of native enzyme is accompanied by anti-UC antibodies production, which prevent uricase activity [9,10]. This problem may probably be prevented by masking the enzyme surface by soluble polymers, a procedure that was found useful to reduce the antigenicity and immuno-

Abbreviations: PEG-1, monomethoxypoly(ethylene glycol) *N*-leucine-OSu; PEG-2, branched monomethoxypoly(ethylene glycol)-OSu; PAcM, poly(*N*-acryloylmorpholine)-OSu; UC, uricase; MRT, mean residue time; AUC, area under the curve.

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genicity and to extend the residence time of enzymes [11–13].

So far polyethylene glycol is the most known of the soluble polymers and a few examples of PEG conjugates approved for human therapy have already reported.

As a further contribution to this methodology, in the present investigation we report the preparation of UC conjugates with the most known linear monomethoxypoly(ethylene glycol) (PEG-1), a branched PEG form PEG-2 and also with the less known poly(*N*-acryloylmorpholine) (PAcM) [14]. The effect of the different polymers in uricase catalytic constants, in the stability of conjugates to heat and to proteolysis as well as the influence of modification on blood residence time were investigated and compared with the properties of the native enzyme. This investigation may also be regarded as a contribution to the understanding of the potentials and limits of poly(*N*-acryloylmorpholine), an alternative polymer to poly(ethylene glycol) recently proposed from our laboratory.

2. Materials and methods

Uricase from *B. fastidiosus* was obtained from Fluka Chemie AG (Buchs, Switzerland).

PEG-1 (M_w 5 kDa) and branched PEG (PEG-2 MW 10 kDa) were obtained from Shearwater Polymers or were prepared by synthesis, as described below. 2,4,6,-Trinitrobenzenesulfonic acid, 4-nitrophenylchloroformate, *N*,*N*-dicyclohexylcarbodiimide, *N*-hydroxysuccinimide and uric acid were purchased from Aldrich Chemie (Steinheim, Germany). *N*-Succinimidyl[2,3-^{3}H]propionate was obtained from Amersham International (Amersham, UK). Trypsin was from Sigma Co. (St. Louis, MO). The organic solvents and salts of analytical grade were from Merck. Male Balb/C mice weighing 25 g and fed 'ad libitum' were used for the pharmacokinetic studies.

For pH titration, a Radiometer autoburette ABU 80 (Copenhagen, Denmark) with titration TTT 80 and titrigraph REA 160 was used. UV–Vis analysis were performed on Perkin–Elmer Lamba2 and Lamba5 spectrophotometers. Analytical SEC separation was carried out with Bio-Gel SEC 30 XL (300 × 7.8 mm) on a Jasco (Tokyo-Japan) HPLC system (880 PU pump, 830 RI detector). Analytical gel filtration chromatography and preparative gel filtration chromatography were performed in a FPLC system (Pharmacia-Uppsala, Sweden) with a Superose 12 TM column.

Protein concentration was determined by the absorption value of the solutions at 280 nm [3] and by the Bio-Rad DC protein assay or according to Lowry procedure [16]. The enzymatic activity of uricase was evaluated spectrophotometrically at room temperature (r.t.), as described by Mahler [3], based on the absorbance decrease at 292 nm in 50 mM borate buffer, pH 9.0 (1 ml) containing 0.059 mM of uric acid using $\varepsilon = 12.2\ mM^{-1}cm^{-1}$. The specific activity is defined as μmol of uric acid converted per min per mg of protein. The assay was linear in the enzyme concentration range 0.086–13.71 μg ml^{-1}. The linearity of the assay was also verified in plasma following the disappearance of uric acid at 292 nm in borate buffer, 0.2 M pH 8.5: to 200 μl of blood, 20 μl of enzymatic solution (2.7–73 μg ml^{-1}) were added and after centrifugation 50 μl were used for the enzymatic activity, as reported above. The degree of modification was determined by titration of the unmodified amino groups with 2,4,6-trinitrobenzenesulfonic acid [17]. Radioactivity was measured using Instagel as liquid scintillation (Packard USA) in a Packard model Minaxi B 4430 liquid scintillation spectrometer.

The amino acid analysis was performed after acid hydrolysis followed by phenylisothiocyanate derivatisation of the amino acids. The evaluation was carried out after reversed-phase HPLC using a Gilson instrument.

2.1. PEG-Nle-OSu (PEG-1) and PEG_2-Lys-OSu (PEG-2)

PEG-Nle-OSu (PEG-1) and PEG_2-Lys-OSu (PEG-2) were prepared as previously reported [15,18]. Briefly, mPEG-OH (M_w 5 kDa), dried with toluene by distillation of the water–toluene azeotrope, activated with 4-nitrophenylchloroformate in dichloromethane at pH 8.0 in the presence of triethylamine (TEA) was bound to 1 equiv. of norleucine (Nle) or lysine (Lys) in aqueous solution at pH 8.0. The degree of functionalization was evaluated by potentiometric titration of the carboxylic group and by amino acid analysis. In case of norleucine, the desired product was directly obtained. In case of lysine ε-monosubstituted lysine was obtained. This intermediate was subsequently PEGylated at the α amino group in anhydrous methylene chloride at pH 8.0 using a small excess of PEG-*p*-nitrophenylcarbonate and fluxing the solution for 3 days. The mixture was concentrated under reduced pressure and precipitated with diethyl ether. The separation of the unreacted products from the excess PEG-*p*-nitrophenylcarbonate and from the hydrolysis products was carried out either by size exclusion chromatography or ion exchange chromatography. PEG-disubstituted Lys of M_w 10 kDa (PEG-2) was characterized by potentiometric titration and ^{1}H NMR. PEG-1 and PEG-2 carboxylic groups were finally activated as succinimidyl ester in anhydrous methylene chloride in the presence of *N*,*N*-dicychlohexylcarbodiimide. The degree of polymer activation was evaluated spectrophotometrically at 260 nm on the basis of the released *N*-hydroxysuccinimide ($\varepsilon_{260\ nm} = 9700\ M^{-1}\ cm^{-1}$) [20].

2.2. PAcM-OSu

The carboxy-terminated poly(*N*-acryloylmorpholine) (PAcM-COOH) used throughout this work was prepared by radical polymerization of *N*-acryloylmorpholine in the presence of 2,2′-azoisobutyronitrile (AIBN) as initiator and 2-mercaptoacetic acid as chain transfer agent [1]. A PAcM–COOH fraction of 6 kDa was obtained from the polydisperse PAcM by precipitation using isopropyl alcohol–dichloromethane (7:3, v/v) as solvent, and ethyl ether as precipitating agent to reach 53% concentration. The molecular weight of the product was determined by sulfur analysis, potentiometric titration of the end-group, and by analytical size exclusion chromatography (Bio-Gel SEC 30XL column using phosphate buffer 0.1 M Na_2SO_4 pH 6.8 as the mobile phase). A calibration curve with polyethylene glycol was used to characterize the PAcM samples of different molecular weights. The polymer was activated as succinimidyl ester, as reported above.

2.3. Enzyme modification

PEG-1 (170 mg) or 200 mg of activated PAcM were added, under rapid stirring, in small portions, at r.t., to 10 mg of uricase dissolved in 4 ml of 0.2 M borate buffer pH 8.5. The amount of PEG-1 or PAcM used in the reaction was 5- or 3-fold molar excess over protein amino groups. The substrate uric acid used as active site protecting agent was eventually present in the reaction mixture in a molar ratio uric acid:uricase (4:1).

After 30 min, the conjugates PEG-1-uricase (PEG-1-UC) and PAcM-uricase (PAcM-UC) were purified from excess of *N*-hydroxysuccinimide, unreacted polymer and uric acid when present, by repeated ultrafiltration on an Amicon system with a PM10 membrane (cutoff 10 000) or XM50 (cutoff 5000). The conjugates were further purified from the unreacted polymer by gel filtration chromatography using a Pharmacia Superose 12 column on a FPLC system eluted with 10 mM phosphate buffer, 0.15 M NaCl (pH 7.2).

For the covalent binding of branched PEG (PEG-2) to uricase to obtain PEG-2-UC, 150 mg of activated polymer (3-fold molar excess with respect to the protein amino groups) were added to 10 mg of uricase, dissolved in 1 ml of borate buffer 0.2 M pH 8.5 under the reaction and fractionation conditions as described above.

2.4. Enzymatic constants

The enzyme activity of native uricase, PEG-1-uricase, PEG-2-uricase and PAcM-uricase in the presence or absence of the competitive inhibitor oxonic acid (0.8–1.6 μg ml^{-1}) was evaluated spectrophotometrically in borate buffer (pH 9.0) at an uricase concentration, expressed as free enzyme, of 2.5 μg ml^{-1} [21]. Uric acid was used as substrate in the concentration range 5.6–72 μg ml^{-1}. The kinetic parameters K_m, V_{max} and K_i were obtained from Lineweaver–Burks plots based on uric acid disappearance rate evaluated at 292 nm.

2.5. Stability to temperature, pH and proteolytic digestion

Thermal stability of native and modified uricase was evaluated on the basis of the residual enzyme activity of protein samples (0.4 mg ml^{-1} in 0.01 M phosphate buffer, 0.15 M NaCl, pH 7.2) heated for 15 min in closed vials at scheduled temperatures (20–80°C) and cooled to r.t.

For the stability to pH, samples of native and modified uricase (0.1 mg ml^{-1} expressed as free enzyme) were dissolved at r.t. in the following buffers: 0.05 M sodium acetate (pH 4–6), 0.05 M phosphate (pH 7), and 0.05 M sodium borate (pH 8–11). After 24 h incubation the enzyme activity was evaluated.

For the stability of uricase to proteolysis, samples of native or modified uricase (0.1 mg ml^{-1}) expressed as free enzyme in 1 ml of 0.05 M phosphate buffer (pH 7.0) were incubated at 37°C with 1.8 mg of trypsin. Aliquots were taken at various time intervals, chilled and assayed for residual enzymatic activity.

2.6. Pharmacokinetic studies

Native uricase (2 ml, 2.5 mg ml^{-1}), PEG-1-UC (2.3 mg ml^{-1}, expressed as protein content), PEG-2-UC (2.6 mg ml^{-1}, expressed as protein content) and PAcM-UC (2.1 mg ml^{-1}, expressed as protein content) in borate buffer 0.2 M pH 8.0 were labelled under mild conditions with 10 μl of toluene containing 25 μg of *N*-succinimidyl[2,3-^{3}H]propionate (^{3}H-NSP) (105 Ci $mmol^{-1}$). This is a known acylating reagent for introducing tritium into proteins without significant loss of enzymatic activity [19]. The solution was vigorously stirred at r.t. for 30 min and the radiolabelled samples purified from the excess of reagent (^{3}H-NSP) by ultrafiltration using a XM50 membrane with a buffer solution 0.05 M sodium phosphate, 0.15 M NaCl (pH 7.2). Under such procedures, products with a specific radioactivity of 0.526, 0.692, 1.033 and 0.482 μCi mg^{-1} were obtained for [^{3}H]UC, [^{3}H]PEG-1-UC, [^{3}H]PEG-2-UC and [^{3}H]PAcM-UC, respectively. Native or modified uricase (150 μl, 2.5 mg ml^{-1}) (expressed as protein content) was intravenously injected into two groups of five Male Balb/C mice weighing about 25 g. Blood (250 μL) was taken at different times from the animal heart or retrobarbital plexus with a heparinized syringe under diethyl ether anaesthesia. The plasma, separated by centrifugation at 3500 rpm for 3 min, was diluted 1:1 with 0.2 M borate buffer (pH 8.5) and the

Table 1
Conditions of reaction, extent of enzyme modification and residual activity of uricase polymer conjugated forms[a,b]

Final conjugate	Ratio between polymer chains and available enzyme $-NH_2$	Degree of NH_2 modification (%)		Residual enzymatic activity (%)
		(a)	(b)	
PEG-1-UC	1:5	41	46	0
PEG-1-UC (site pr.)	1:5	44	40	40
PEG-2-UC	1:3	46	35	76
PAcM-UC	1:3	22		0
PAcM-UC (site pr.)	1:3	18		24

[a] The extent of modification was calculated on the basis of unconjugated amino groups by the trinitrobenzene sulfonate colourimetric assay [17].
[b] The extent of modification was calculated on the basis of the Nor-leucine or lysine content calculated by amino acid analysis after acid hydrolysis [23]. This procedure is not suitable to PAcM conjugate since in this case no reporter amino acid is present in the polymer. Site pr., active site protection with uric acid.

enzyme content evaluated by ^{3}H-radioactivity. Uricase determinations, carried out both in 0.2 M borate buffer (pH 8.5) and in plasma, showed the same linearity range (0.08–3.6 μg ml^{-1}). The enzyme content in plasma was also evaluated by enzymatic acticity, as reported above. The plasma half-life elimination ($t_{1/2}\beta$), AUC, MRT and k values were obtained by the plasma values according to standard procedures.

3. Results

In Table 1 the degree of modification and the residual activity of uricase following reaction with linear PEG (PEG-1), branched PEG (PEG-2) and PAcM are reported. The table shows that the reaction with PEG-1 and with PAcM is accompanied by complete loss of enzyme activity, that however could be prevented by carrying out the reaction in the presence of a large excess of substrate as active site protection. On the contrary, active site protection was not needed when PEG-2 was used in uricase conjugation. This behaviour is similar to what was already observed in our laboratory in asparaginase modification by the two PEGs. Most probably this is explained by the different hindrance of the two polymers that prevents, in the case of PEG-2 conjugate, to reach the active site cleft which, in case of uricase, is between the non-symmetrical subunits [22].

Table 2 reports the enzymological parameters of native and polymer modified uricase. The table shows that the K_m values do not change upon conjugation. On the other hand, a large increase in the oxonic acid inhibition takes place in the conjugated forms that, in the case of PAcM-UC, is 100 times higher than that of the native enzyme. This behaviour, although surprising, may be explained by the loss of enzyme amine positive charges following acylation with the carboxylic activated polymers which, if present in the surroundings of the active site, would result in decreased attraction of the negatively charged oxonic acid inhibitor.

The stability of uricase conjugates to trypsin digestion is reported in Fig. 1. In the figure, the digestion of the native enzyme is reported as a dotted line since it cannot be directly compared with the conjugated enzymes. Native uricase, in contrast to modified forms, has all the lysines available as potential digestion sites, while the conjugated enzyme loses, by acylation, part of the positive charge of this residue, thus reducing the

Table 2
Kinetic constants of uricase and uricase conjugates [a]

	K_m ($\times 10^{-5}$ M)	V_{max} 10^{-3}	K_i ($\times 10^{-7}$ M)
Native UC	5	2.0	0.5
PEG-1-UC (site pr.)	5	0.6	4.5
PEG-2-UC	5	1.3	5.8
PacM-UC (site pr.)	4.2	1.0	4.2×10^{-6} M

[a] Site pr, active site protection with uric acid.

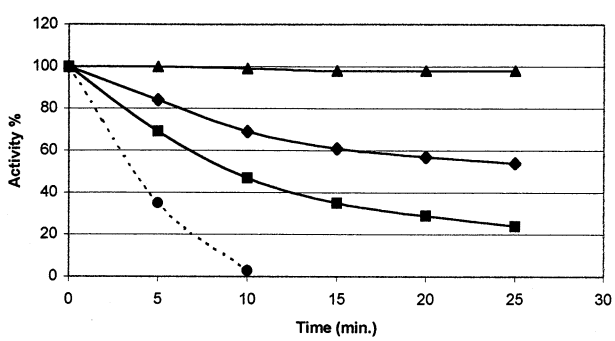

Fig. 1. Resistance to proteolysis of uricase conjugates. Kinetics of enzyme activity loss of native or conjugated uricase forms following trypsin incubation. PAcM-UC (♦), PEG-1-UC (■), PEG-2-UC (▲) and native uricase (●).

Table 3
Relevant pharmacokinetic parameters of uricase and uricase conjugates[a]

	$T_{1/2}$ β	AUC_{∞} (mg ml^{-1} per min) ($\times 10^5$)	MRT (min)	K
Native uricase	390	8×10^4	470	3.8×10^{-3}
PAcM-UC	1190	2.2	1590	1.3×10^{-3}
PEG-1-UC	3384	6.1	3930	4.3×10^{-4}
PEG-2-UC	2564	6.8	3450	5×10^{-4}

[a] Pharmokinetic parameters of native or conjugate uricase evaluated in mice plasma following administration of ^{3}H labelled products. The blood was withdrawn at suitable time intervals by retrobulbar eye puncture.

hydrolysis rate. In any case the comparison holds among linear PEG, branched PEG and PAcM conjugates since all of them undergo this phenomenon. The greatest effect in enzyme protection by branched PEG conjugation and the good protection of PAcM, although, in this case, a lower number of polymer chains are bound to the enzyme (see Table 1).

Thermostability studies carried out by 15 min incubations at different temperatures demonstrated that both native and conjugated samples are completely inactivated at 65–67°C, although different inactivation rates were observed. The temperature of 50% inactivation was found at 60–61°C in native uricase and PAcM-uricase, while at 52°C in the linear or branched polymers conjugated samples. This behaviour of thermal inactivation of uricase indicates a destabilizing effect by polymer conjugation that was already observed with other enzymes. The effect, in case of uricase, is related to the structure of the polymer and is higher for the PEG-1 and PEG-2 derivatives.

Table 3 reports relevant pharmacokinetic parameters of native and polymer conjugated uricase species: the data demonstrate longer blood residence time of the conjugated forms, although the residence time differs from one another. The longest mean residence time (MRT) was found in PEG-1-UC (MRT = 3930 min) followed by PEG-2-UC (MRT = 3450 min) and PAcM-UC (MRT = 1590 min), while the MRT of native uricase was 470 min only. These values of blood residence time are in good agreement with other pharmacokinetic parameters reported in the table, namely elimination constants and $T_{1/2}\beta$.

4. Discussion

The results of uricase modification described in this paper are important in view of a safer use of the enzyme in therapy. The paper demonstrates also new possibilities for enzyme derivatisation, in particular it shows that poly(*N*-acryloylmorpholine) may be employed as a soluble monofunctional oligomer for protein conjugation.

PAcM was recently proposed by our laboratory as an alternative to PEG, but few data have been reported so far on its use. The results reported here show that the PAcM-uricase conjugate, as compared with free uricase, exhibits improved stability to proteolysis, higher structural stability and longer blood residence time. In this regard we recall the findings obtained in liposome modification by PAcM: the PAcM conjugated liposomes were long lasting in blood as were those modified by linear or branched PEG [24].

For a more complete description of PAcM as a polymer suitable for conjugation, it may also be useful to remember the comparison between PEG and PAcM lipase for bio-conversion in organic solvents. A thorough study already demonstrated that the former conjugate possesses higher solubility in chlorinated solvents, while the latter exhibits a higher transesterification rate [25]. Furthermore, in the polymer supported liquid phase synthesis of oligonucleotides, PEG was found to be superior for conveying solubility in organic solvents and also favorable for its spectral properties, while PAcM presented the advantage of higher rate in detritilation [26].

All of these data, taken together, demonstrate that not one polymer does exist that may be considered 'the best' for conjugation, but that any one, linear PEG, branched PEG or PAcM and so on, have properties suitable for different applications.

In addition, an important result that comes from this research is the high degree of residual uricase enzymatic activity observed after conjugation with branched PEG. In fact, in the case of linear polymers, either PEG or PAcM, a high degree of activity retention was found only if an excess of substrate was present in the enzyme modification mixture as active site protection. This behaviour, in agreement with what was recently observed in our laboratory in asparaginase modification, demonstrates that this favourable property of branched PEG may be general. It is reasonable to think that this occurs when the enzyme active site is located, as in the case of this enzyme, in a narrow cleft where the hindered branched PEG cannot enter, and furthermore when at the active site, or at its surrounding, amino groups reactive towards the polymer are present.

In view of the therapeutic application of uricase, the finding of blood residence time evaluated in mice must be reported. The uricase residence time of the conjugated forms, calculated as $T_{1/2}$ elimination time or as elimination constants or MRT, are in the following rank order of favour: PEG-1-UC > PEG-2-UC > PAcM-UC > UC. AUC, a parameter of paramount importance for therapy, shows a similar behaviour since it is from three to eight times higher than that of the unconjugated enzyme.

In conclusion, the data reported in this paper, taken together, are in favour of polymer conjugation for an improved therapy with uricase. Furthermore, the results reported here add new information regarding the properties of PAcM, a polymer that so far is not greatly employed for this application. More in vivo data on this polymer, as well as on a new end functionalized form of PVP, regarding organ distribution and immunogenicity are now being obtained in our laboratory and will be reported soon.

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